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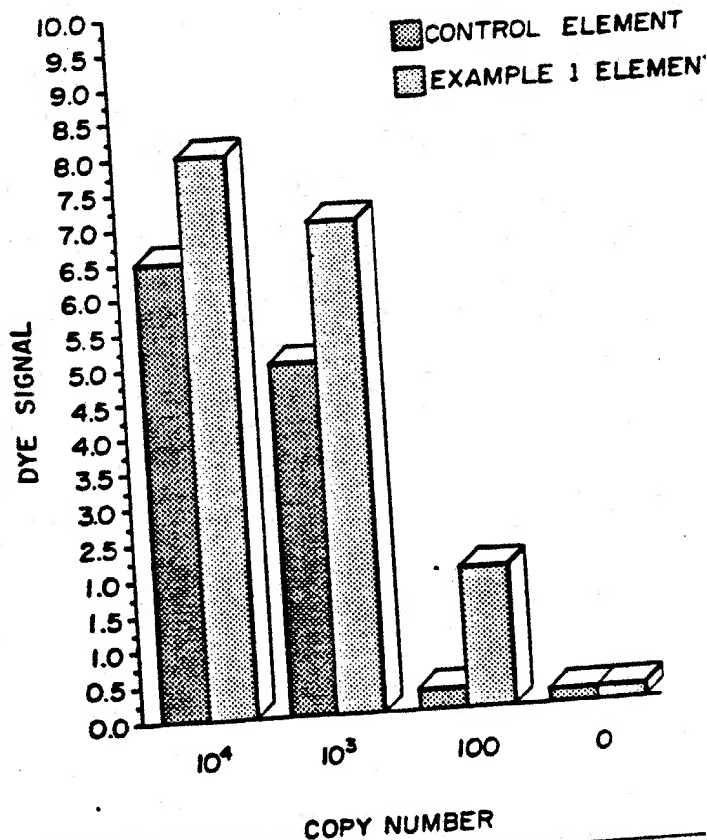
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| (71) Applicant: EASTMAN KODAK COMPANY [US/US]; 343 State Street, Rochester, NY 14650 (US). | | | | |
| (72) Inventors: SEABERG, Leonard, Joseph; 46 Preview Drive, Penfield, NY 14526 (US). SCHNIPPELSKY, Paul, Nicholas; 89 Canterbury Road, Rochester, NY 14607 (US). HINCKLEY, Charles, Cullis; 23 Latchmere Court, Pittsford, NY 14650 (US). WELLMAN, Jeffrey, Allen; 1544 Stone Road, Rochester, NY 14615 (US). DONISH, William, Harold; 320 Sweet Birch Lane, Rochester, NY 14615 (US). FINDLAY, John, Bruce; 148 Crossroads Lane, Rochester, NY 14612 (US). SUTTON, Richard, Calvin; 24 Twilight Drive, Rochester, NY 14617 (US). PONTICELLO, Ignazio, Salvatore; 21 Copper Woods, Pittsford, NY 14534 (US). CUMMINS, Thomas, Joseph; 176 Elmcroft Road, Rochester, NY 14609 (US). ZANDER, Dennis, Roland; 35 Rolling Meadows Way, Penfield, NY 14526 (US). | | Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments. | | |

(54) Title: ELEMENT AND METHOD FOR NUCLEIC ACID AMPLIFICATION AND DETECTION USING ADHERED PROBES

(57) Abstract

An element has been prepared which is useful for the detection of nucleic acids in various formats. The element has a sealable support on which is disposed a nucleic acid reagent composition. The composition is a mixture of a nucleic acid reagent composed of polymeric particles to which an oligonucleotide is covalently attached. The particles are prepared from a first polymer having a glass transition temperature of at least 70 °C and have an average diameter of from 0.1 to 3 micrometers. The reagent is adhered to the support using a water insoluble adhesive comprising a second polymer which has a glass transition temperature which is at least 30 °C less than the glass transition temperature of the first polymer. The adhesive is present in the composition at from 1 to 20 weight percent. This element provides high sensitivity and low background in hybridization and other nucleic acid assays.



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ELEMENT AND METHOD FOR NUCLEIC ACID AMPLIFICATION AND
DETECTION USING ADHERED PROBES

This invention relates to an element having an oligonucleotide attached thereto, a method of making the
5 element and a diagnostic kit and method for its use. This invention is particularly directed to the field of nucleic acid diagnostics.

Nucleic acid probe technology has developed rapidly in recent years as researchers have discovered its
10 value for detection of various diseases, organisms or genetic features which are present in small quantities in a human or animal test sample. The use of probes is based upon the concept of complementarity. DNA has two strands bound together by hydrogen bonds between complementary
15 nucleotides (which are also known as nucleotide pairs).

The DNA complex is normally stable, but the strands can be separated (or denatured) by conditions which disrupt the hydrogen bonding. The released single strands will reassociate only with another strand having a
20 complementary sequence of nucleotides. This hybridization process can occur with both strands being in solution or with one of the strands being attached to a solid substrate.

A targeted nucleic acid sequence in an organism or cell may be only a very small portion of the entire DNA
25 molecule so that it is very difficult to detect its presence using most labeled DNA probes. Much research has been carried out to find ways to detect only a few molecules of a target nucleic acid.

A significant advance in the art is described in
30 US-A-4,683,195, US-A-4,683,202 and US-A-4,965,188. These patents describe amplification and detection methods wherein primers are hybridized to the strands of a target nucleic acid (considered the templates) in the presence of a nucleotide polymerization agent (such as a DNA polymerase)
35 and deoxyribonucleoside triphosphates. Under specified

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conditions, the result is the formation of primer extension products as nucleotides are added along the templates from the 3'-end of the primers. These products are then denatured and used as templates for more of the same primers in another extension reaction. When this cycle of denaturation, hybridization and primer extension is carried out a number of times (for example 25 to 30 cycles), the process which is known as "polymerase chain reaction" (or PCR) exponentially increases the original amount of target nucleic acid so that it is readily detected.

Once the target nucleic acid has been sufficiently amplified, various detection procedures can be used to detect it, as noted in the cited patents.

Various devices have been designed for hybridization assays whereby a target nucleic acid is insolubilized prior to detection using appropriate detection probes. For example, nitrocellulose filters and other planar, solid supports have been used for this purpose as described for example, in US-A-4,925,785.

One technique used to attach probes or other reagents is merely to dry them down onto the solid support, as described for example in EP-A-0 381 501. More recently, probes attached to polymeric particles are fused into a solid support formed of another polymer which can be softened by heat.

While providing improvement over prior techniques, immobilization of capture reagents (such as capture probes) by drying or fusing has limitations. Adhesion of the reagents is poor. Particularly in assays whereby reagents in solution are washed over the immobilized capture probes, some of the probes are dislodged and potential signal is lost when the probes are washed away. Moreover, fusion of capture probes to a solid support requires high temperatures and dangerous equipment which tend to reduce the ability of the probes to capture a target nucleic acid.

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It would be desirable to avoid the problems associated with known means for immobilizing capture probes while providing highly sensitive assays with minimal loss in signal associated with loss of capture probe. —

5 The problems noted above have been solved with an element comprising a sealable support having disposed thereon a nucleic acid reagent composition comprising a mixture of:

10 a. a nucleic acid reagent comprising:
particles composed of a first polymer having a glass transition temperature of at least 70°C, the particles having an average diameter of from 0.1 to 3 micrometers, and
an oligonucleotide covalently attached to the
15 particles, and

 b. a water insoluble adhesive comprising a second polymer which has a glass transition temperature which is at least 30°C less than the glass transition temperature of the first polymer,
20 the polymeric adhesive being present in the composition at from 1 to 20 weight percent.

Moreover, this invention also provides a self-contained test device comprising the element described above.

25 Further, a method for preparing a nucleic acid test element comprises:

A. depositing a nucleic acid reagent composition on a sealable support, the composition comprising a mixture of:

30 a. a nucleic acid reagent comprising:
particles composed of a first polymer having a glass transition temperature of at least 70°C, the particles having an average diameter of from 0.1 to 3 micrometers, and
an oligonucleotide covalently attached to the
35 particles, and

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b. a water insoluble polymeric adhesive comprising a second polymer which has a glass transition temperature which is at least 30°C less than the glass transition temperature of the first polymer,

5

the polymeric adhesive being present in the composition at of from 1 to 20 weight percent, and

B. heating the deposited nucleic acid reagent composition at a temperature and for a time sufficient to adhere the composition to the support and to dry it.

10

This invention also provides a diagnostic test kit comprising:

(1) the element described above, and
(2) a detection reagent for detecting the hybridization product of the nucleic acid reagent oligonucleotide and the target nucleic acid.

15

Still further, a method for the detection of a target nucleic acid comprises:

A. contacting a specimen suspected of containing a target nucleic acid with the element described above wherein the oligonucleotide of the disposed nucleic acid reagent is complementary to the target nucleic acid,

20

to form a hybridized product of the target nucleic acid and the nucleic acid reagent on the element, and

25

B. detecting the presence of the hybridized product as an indication of the presence of the target nucleic acid in the specimen.

Moreover, also provided is a method for the amplification and detection of a target nucleic acid comprising:

30

A. amplifying a target nucleic acid in a test specimen,

B. contacting the amplified target nucleic acid with the element described above wherein the oligonucleotide of

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the disposed nucleic acid reagent is complementary to the target nucleic acid,

to form a hybridized product of the amplified target nucleic acid and the nucleic acid reagent on the element,
5 and

C. detecting the presence of the hybridized product
- as an indication of the presence of the target nucleic acid in the specimen.

The present invention provides an improved element
10 which is useful in the detection of nucleic acids. High signal and low background are features of the improved element and method for its use. It is particularly useful for the detection of nucleic acids which are present in very low quantities, for example, as low as 100 molecules, in a
15 specimen. This invention provides an improved element for diagnostic use whereby the capture probe is deposited in defined areas and is not easily dislodged by fluids or mechanical handling. Thus, the potential signal from the capture of the target nucleic acid is well defined and
20 exhibits high density.

These features are achieved with an element which has the appropriate nucleic acid reagent firmly attached to a solid support in the element. The disadvantages associated with reagent fusing and drying are avoided, and
25 the reagents are not washed away during an assay.

The element has these important advantages as a result of the use of a particular combination of polymers and adhesive to attach the particulate nucleic acid reagent to a solid sealable support. The amount of adhesive used is
30 adjusted in relation to the amount of reagent present so the reagent and targeted nucleic acid can readily come into contact. The reagent has particularly small particles in order to provide high sensitivity and optimum adhesion to the support.

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The FIGURE is a graphical representation of the results of comparative assays performed as described in Example 2 below.

5 The element of this invention comprises a sealable support having disposed thereon (as described below) one or more nucleic acid compositions, each comprising a nucleic acid reagent in admixture with a water insoluble adhesive.

10 Sealable supports are resinous materials (usually synthetic polymers) which are capable of being sealed (or fused) to themselves or to another sheet of material using heat or ultrasonic pressure sealing techniques. Preferably, the supports are heat-sealable. Useful sealable supports include, but are not limited to, sheets, fibrous mats, and membranes which can be sealed to themselves in an
15 appropriate manner and in appropriate places to provide channels or voids between sealed sheets, mats or membranes. It is also preferred that the support surface is roughened in some fashion to facilitate adhesion of the nucleic acid composition.

20 The supports can be composed of, for example, polyesters (such as poly(ethylene terephthalate), poly[4,4'-(hexahydro-4,7-methanoindan-5-ylidene)diphenylene terephthalate] and poly(4,4'-isopropylidenediphenylene 1,1,3-trimethyl-3-phenyl-5,4'-indandicarboxylate)), polycarbonates
25 [such as biphenol A polycarbonate (for example, LEXANTM sold by General Electric)], polyamides [such as poly(p-phenylene terephthalamide)], polyimides [such as the polyimide product of 4,4'-diaminodiphenylether and pyromellitic dianhydride], and celluloses [such as cellulose acetate, and cellulose
30 acetate butyrate].

In one embodiment, sheets of polyethylene are sealed at the peripheral edges to form a container having voids for various reagents, including the nucleic acid reagents described herein. In another embodiment, laminates
35 of polyethylene and a polyester, such as poly(ethylene

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terephthalate), can be heat sealed. Laminates can have a variety of layers therein including adhesives or vapor barriers as well as sealable layers. Other embodiments would be readily apparent to one skilled in the art.

5 The nucleic acid reagent described herein is deposited on a surface of the sealable support, uniformly thereover, or in one or more defined regions. Preferably, the reagent is in a defined region of the support surface such as in a round spot, printed stripe or other desired
10 configuration. In one embodiment, several different nucleic acid reagents are deposited in separate defined regions of the support surface so that a multiplicity of target nucleic acids can be simultaneously detected using the element. For example, the element can have a series of reagent spots or
15 stripes.

 The nucleic acid reagent useful in this invention is composed of particles of a first polymer (or composite of polymers) having a glass transition temperature (T_{g1}) of at least 70°C. Preferably, the T_{g1} is from 70 to 175°C, and
20 more preferably it is in the range of from 75 to 140°C. Glass transition temperature refers to the temperature at which the polymer changes from a glassy state to a rubbery, flowing or tacky polymer. Procedures for measuring glass transition temperatures are described in Techniques and
25 Methods of Polymer Evaluation, Vol. 1, Marcel Dekker, Inc., New York, 1966.

 The particles useful herein are also impermeable and non-swellaable in aqueous fluids. These properties insure structural integrity for the composition disposed on
30 the sealable support of the element. Non-swellaability refers to particles exhibiting little swell (that is, less than 10% swell) in an aqueous fluid as measured using a swellometer of the type described by Green et al, J. Photo. Sci., 20, 205 (1972), after immersion of the
35 particles in an aqueous fluid at 38°C for 2.5 minutes.

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Preferably, the particles are spherical in shape although other shapes can be used as well as long as the largest dimension of the particles is no greater than the maximum diameter for spherical particles as described below.

5 It is also preferred that the particles be substantially uniform in size. Critically, the largest particle dimension (for example, diameter) is from 0.1 to 3 μm , but a diameter in the range of from 0.5 to 2 μm is preferred.

The particles are generally composed of, at least
10 on the surface thereof, naturally occurring or synthetic materials to which an oligonucleotide can be covalently attached (described below). Such materials generally have reactive groups with which the oligonucleotide or a derivatized form thereof can be reacted to form a covalent
15 bond.

In general, any reactive group with which an amino or sulfhydryl group is reactive is useful in this context. Particularly useful reactive groups include, but are not limited to, an active halogen, carboxy, amidine, activated
20 2-substituted ethylsulfonyl, activated 2-substituted ethylcarbonyl, vinylsulfonyl, vinylcarbonyl, epoxy, aldehyde, sulfhydryl, amino (after activation), hydrazine and active esters such as succinimidoxycarbonyl. Preferred particles are organo-polymeric beads such as those described
25 in EP-A-0 323 692 prepared from one or more ethylenically unsaturated polymerizable monomers having an active halogen, activated 2-substituted ethylsulfonyl or vinylsulfonyl groups. Most preferred particles have reactive carboxy groups, as described in EP-A-0 466 220 which are prepared
30 from monomers having the general structure



wherein R is hydrogen, halo or alkyl of 1 to 3 carbon atoms, M is hydrogen, an alkali metal ion or an ammonium ion and L is an organic linking group having from 8 to 50 carbon,
35 oxygen, nitrogen or sulfur atoms in the linking chain.

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Representative useful ethylenically unsaturated polymerizable monomers having reactive groups include, but are not limited to, acrylic acid, methacrylic acid, m & p-(2-chloroethylsulfonyl-methyl)styrene, m & p-[2-(p-tolylsulfonyloxy)ethyl-sulfonylmethyl]styrene, m & p-vinylsulfonylmethyl-styrene, N-[m & p-(2-chloroethylsulfonylmethyl)-phenyl]acrylamide, N-[2-(2-chloroethylsulfonyl)ethyl-formamidomethyl]acrylamide, mono-m & p-vinylbenzyl glutarate, mono-p-vinylbenzyl glutarate, 10 mono-2-methacryloyloxyethyl glutarate, 2-(4-carboxybutyramido)ethyl methacrylate, 2-[N'-(5-carboxypentyl)-ureido]ethyl methacrylate, mono-methacryloylpenta(oxyethylene) glutarate, mono-(4-acryloyloxybutyl) glutarate, 4-(4-carboxybutyramido)styrene, mono-2-(p-vinylbenzylthio)ethyl glutarate, mono-2-(m & p-vinylbenzylthio)ethyl glutarate, 4-(4-carboxybutyramidomethyl)styrene, mono-2-[N-methyl-N-(4-vinylbenzyl)amino]ethyl glutarate, 3-(p-vinylbenzylthio)-propionic acid, 4-[2-(4-carboxybutyramido)ethyl-thiomethyl]styrene, 4-[2-(carboxymethoxyacetamido)-ethylthiomethyl]styrene, 4-[2-(carboxymethylthioacetamido)ethylthiomethyl]styrene, mono-2-(p-vinylbenzylthio)ethyl succinate, 4-[2-(carboxymethoxyacetoxymethylthio)ethylthiomethyl]styrene, mono-4-vinylbenzyl succinate, 25 2-(4-vinylbenzylthio)succinic acid, 2-(4-vinylbenzylthio)benzoic acid, mono-2-[2-(4-vinylbenzylthio)ethoxy]ethylthiomethyl malonate, mono-methacryloylpenta(oxyethylene) phthalate, mono-2-[2-(4-vinylbenzylthio)ethoxy]ethylthio)ethyl succinate, mono-2-[2-[2-(4-vinylbenzylthio)ethoxy]ethylthio)ethyl phthalate, 30 [2-(4-vinylbenzylthio)ethoxy]ethylthio)ethyl phthalate, 3-[4-(4-vinylbenzyloxy)benzylthio]propionic acid and 4-[4-(4-vinylbenzyloxy)benzylthio]benzyl-thio)butyric acid.

The monomers just described can be polymerized as homopolymers, but preferably they are copolymerized with one

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or more other ethylenically unsaturated polymerizable monomers.

More particularly, the particles useful in this invention are composed, at least on the surface thereof, of a polymer comprising:

(a) from 0.1 to 60 weight percent of recurring units derived from one or more ethylenically unsaturated polymerizable monomers having a reactive group as defined above,

(b) from 40 to 99.9 weight percent of recurring units derived from one or more ethylenically unsaturated polymerizable monomers which, when homopolymerized, provide a water-insoluble homopolymer, and

(c) from 0 to 15 weight percent of recurring units derived from one or more ethylenically unsaturated polymerizable monomers other than those defined for components (a) and (b) above, including but not limited to hydrophilic monomers which provide colloidal stability to the copolymer.

Useful monomers for component (b) noted above include, but are not limited to, vinyl aromatics (for example, styrene and styrene derivatives such as 4-vinyltoluene, α -methylstyrene, 2,5-dimethylstyrene, 4-*t*-butylstyrene and 2-chlorostyrene), acrylic and methacrylic acid esters and amides (for example, methyl acrylate, methyl methacrylate, *n*-butyl acrylate, 2-ethylhexyl methacrylate, benzyl acrylate and *N*-phenylacrylamide), butadiene, acrylonitrile, vinyl acetate, vinylbenzyl acetate, vinyl bromide, vinylidene chloride and crosslinkable monomers having two or more polymerizable groups (such as vinyl groups, for example divinylbenzene, and di- and triacrylates). Other monomers which are capable of providing a water-insoluble homopolymer would also be useful, and would be readily apparent to one skilled in the

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art. The vinyl aromatic monomers are preferred for component (b).

Useful monomers for component (c) include, but are not limited to, nonionic hydrophilic monomers such as
5 acrylamide, methacrylamide, N-isopropylacryl-amide, 2-hydroxyethyl acrylate, 2-hydroxyethyl meth-acrylate, N-vinyl-2-pyrrolidone and others readily apparent to one skilled in the art. In addition, mono-mers having active methylene groups, such as 2-aceto-acetoxyethyl methacrylate,
10 and cationic monomers, such as N,N,N-trimethyl-N-vinylbenzylammonium chloride and 3-hydroxyethyl-1-vinylimidazolium chloride could be used.

Still other useful monomers for component (c) include those having polyoxyalkylene side chains as
15 described for example in US-A-5,086,143. Representative monomers, include but are not limited to, pentaethylene glycol monomethacrylate, decaethylene glycol monomethacrylate, eicosaethylene glycol monomethacrylate, pentaethylene glycol monoacrylate, polypropylene glycol
20 monomethacrylate and polypropylene glycol monomethacrylate.

A preferred monomer for component (c) is 2-acetoacetoxyethyl methacrylate.

Mixtures of various monomers for each component (a), (b) and (c) can be copolymerized as long as the
25 monomers are compatible with each other and sufficient reactive groups are present on the surface of the resulting particles.

Preferred first addition polymers include poly[styrene-co-mono-3-(p-vinylbenzylthio)propionic acid] or
30 poly[styrene-co-mono-2-(p-vinylbenzylthio)ethyl succinate].

Preferably, the particles are composed of recurring units derived by addition polymerization from 2 to 15 weight percent of component (a), from 85 to 98 weight percent of component (b), and from 0 to 10 weight percent of
35 component (c). More preferably, they are composed of

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recurring units derived from 2 to 10 weight percent of (a), from 90 to 98 weight percent of component (b), and from 0 to 10 weight percent of component (c).

5 The copolymers useful herein to make the particles are prepared using standard emulsion or suspension polymerization techniques.

10 The particles can also be core/shell particles having the noted polymer described above as the shell so that reactive groups are available on the surface. The core of such particles can be composed of any suitable polymer which contributes to the requisite physical integrity and glass transition temperature and is generally different from that of the shell polymer.

15 Molecules of an oligonucleotide are covalently attached to the particles. The term "oligonucleotide" refers to a molecule comprised of two or more (preferably more than three) deoxyribonucleotides or ribonucleotides. Its size will depend upon many factors including the target nucleic acid to which it is complementary, the size of the
20 polymeric particle to which it is attached, and the means of attachment to the particles including the length of any spacer molecule.

The oligonucleotide is covalently attached to the polymeric particles using any suitable technique. They can
25 be directly attached by reacting the reactive groups on the surface of the particles with corresponding sulfhydryl, carboxy or amino groups of the oligonucleotide. Alternatively, the oligonucleotide can be biotinylated or otherwise modified to add a specific binding species which
30 can then specifically bind with its corresponding receptor which can be attached to the particles. Avidin-biotin complexes are known to be used for this purpose as described for example in EP-A-0 139 489, EP-A-0 192 168 and EP-A-0 370 694. Incorporating biotin into an oligonucleotide can be

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achieved using known technology including that described in EP-A-0 097 373 (published January 4, 1984).

Preferably, however, it is desired to chemically modify the oligonucleotide in order to provide reactive groups therein or to provide "spacer" groups or "linker" groups to extend the oligonucleotide away from the surface of the particles. Techniques for doing this are well known, as described for example, in US-A-4,914,210 and WO 89/11548.

A polymeric adhesive is used to affix the nucleic acid reagent of the nucleic acid composition to the sealable support of the element. It also acts to bond the reagent particles to each other. This adhesive comprises a second polymer which has a glass transition temperature (T_{g2}) which is at least 30°C less than the glass transition temperature (T_{g1}) of the first polymer of the particles. Preferably, T_{g2} is from 30 to 120°C less than T_{g1} . T_{g2} is also less than 90°C. More preferably, T_{g2} is in the range of from -50 to +40°C.

The adhesive is insoluble in aqueous fluids commonly encountered in diagnostic and analytical methods. While it is not essential, it is also preferred that the adhesive be non-swellaable in aqueous fluids.

More particularly, the second polymer is composed of:

(d) from 55 to 100 weight percent of recurring units derived from one or more ethylenically unsaturated polymerizable monomers used in component (b) of the first polymer described above,

(e) from 0 to 45 weight percent of recurring units derived from one or more ethylenically unsaturated monomers which form a water soluble homopolymer, or which provide hydrophilicity from polar groups [such as primary, secondary and tertiary amines, hydroxy and poly(alkyleneoxy) groups], anionic groups [such as carboxylate, sulfonate, sulfate, phosphate and phosphonate], and cationic groups [such as

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trialkylammonium and trialkylphosphonium] and others readily apparent to one skilled in the art, and

- (f) from 0 to 15 weight percent of recurring units derived from one or more ethylenically unsaturated monomers which provide crosslinking in the polymer adhesive.

While the monomers described above for component (b) of the first polymer are useful also in component (d), the preferred monomers for component (d) are alkyl acrylates and methacrylates wherein the alkyl group has from 1 to 8 carbon atoms (such as methyl, ethyl, *n*-propyl, *n*-butyl, isobutyl, 2-ethylhexyl, hexyl and octyl), and which alkyl group can also be interrupted with one or more thio, oxy or iminoalkyl groups having 1 to 6 carbon atoms. More preferred monomers include, but are not limited to, methyl acrylate, methyl methacrylate, *n*-butyl acrylate and *n*-butyl methacrylate. Methyl acrylate is most preferred.

Useful monomers for component (e) include, but are not limited to, charged monomers (cationic or anionic) such as acids and salts thereof, including but not limited to, acrylic acid, methacrylic acid, itaconic acid, 2-acrylamido-2-methylpropanesulfonic acid, 3-methacryloyloxypropane-1-sulfonic acid and their salts, N-(2-acryloyloxyethyl)-N,N,N-trimethylammonium methosulfate, 3-hydroxyethyl-1-vinylimidazolium chloride, aminoethyl-methacrylate hydrochloride, N-(2-aminopropyl)methacrylamide hydrochloride, 2-carboxyethyl acrylate, *p*-styrene sulfonic acid and salts thereof, *m* & *p*-carboxymethylstyrene and its salts, and other ethylenically unsaturated polymerizable sulfonates, carboxylates, sulfates, phosphonates, quaternary ammonium salts, pyridinium salts, imidazolium salts or quinoxalinium salts. Also useful are the carboxy-containing monomers (and salts thereof) described above for component (a) of the first polymer.

Nonionic monomers which also are useful in component (e) include amine-containing monomers, such as

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dimethylaminopropyl acrylate and diethylaminoethyl methacrylate and hydroxy-containing monomers such as 2-hydroxyethyl acrylate, 2-hydroxyethyl methacrylate, 2,3-dihydroxypropyl acrylate and pentaethylene glycol
5 monoacrylate.

Preferred monomers in component (e) include 2-acrylamido-2-methylpropanesulfonic acid and salts thereof, 2-aminoethyl methacrylate hydrochloride and N-(2-aminopropyl)methacrylamide hydrochloride.

10 The monomers for component (f) which provide crosslinking can either be crosslinked during polymerization, or provide crosslinking after subsequent chemical reaction with themselves or with additional crosslinking agents. Such monomers include multifunctional
15 vinyl monomers such as di- and triacrylates and methacrylates (such as ethylene diacrylate and ethylene dimethacrylate), divinylbenzenes, and monomers containing active methylene groups which can be crosslinked using known chemical reactions. Examples of the latter include 2-
20 acetoacetoxyethyl methacrylate, N-(2-acetoacetoxyethyl)acrylamide, N-(2-acetoacetamidoethyl)methacrylamide, 6-(m & p-vinylphenyl)-2,4-hexanedione, ethyl acryloylacetate and others known in the art.

25 Preferred monomers for component (f) include 2-acetoacetoxyethyl methacrylate, N-(2-acetoacetoxyethyl)acrylamide and N-(2-acetoacetamidoethyl)methacrylamide.

30 Preferably, the copolymers useful in preparing the adhesive are composed of recurring units derived from 70 to 98 weight percent of component (d), from 2 to 30 weight percent of component (e), and from 0 to 10 weight percent of component (f). More preferably, they are composed of recurring units derived from 85 to 95 weight percent of

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component (d), from 2 to 15 weight percent of component (e), and from 0 to 8 weight percent of component (f).

A preferred addition polymer for the adhesive is poly[methyl acrylate-co-2-acrylamido-2-methylpropanesulfonic acid, sodium salt-co-2-acetoacetoxyethyl methacrylate].

In the nucleic acid reagent composition comprising a mixture of polymeric adhesive and nucleic acid reagent used in this invention, the adhesive can be present in an amount of up to 20% by dry weight. Preferably, the adhesive is present in an amount of from 1 to 20% by weight, with an amount of from 2 to 10 weight percent being more preferred.

The composition can optionally contain other addenda, such as buffers, surfactants or binders in minor amounts, that is generally less than 5% of the total composition weight.

The nucleic acid reagent composition can be applied to the sealable support in any suitable manner. For example, it can be coated thereon using standard coating equipment and techniques, applied by hand, printed thereon, spotted with a pipette tip, microsyringe tip or microdispensing pump, and then dried. Preferably, however, the composition is merely disposed in an aqueous suspension with a microsyringe tip and dried on a sealable support. Drying is accomplished by heating the disposed composition in a range of from 10 to 80°C less than the glass transition temperature of the first polymer described above.

The coverage of the nucleic acid reagent composition on the support can be varied considerably depending upon the type of assay format, the configuration of the assay element and other factors that one skilled in the art would readily perceive. For example, the composition can be applied uniformly to the sealable support, or applied in defined regions thereof (as noted above). Application of the composition in defined regions is preferred. Techniques for applying different reagents to

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different regions are well known. In some instances, the reagents can be disposed in defined regions that provide visual patterns for easy detection.

It is preferred to use a sealable support which
5 has been pretreated to render it more hydrophilic. The
- nucleic acid reagent composition is adhered more effectively
to the support in such embodiments. The pretreatments can
be of a chemical, electrical or mechanical nature, or a
combination of different types of treatments. For example,
10 chemical treatments include the use of chromic acid which
involves etching a surface with sodium dichromate in
sulfuric acid for a few minutes.

The support can also be treated with activated
species in gases, such as noble gases. Still another known
15 procedure is the use of nitrous oxide at elevated
temperatures.

Electrical treatments include corona discharge,
flaming and electrode discharge processes.

Another pretreatment involves simultaneous
20 chemical and electrical treatment such as with a radio
frequency electromagnetic field in the presence of a
reactive gas.

Preferred pretreatments include corona discharge
treatment, chromic acid treatment and treatment with a radio
25 frequency electromagnetic field in the presence of a
reactive gas. Corona discharge treatment is most preferred.

Alternative or supplemental to the pretreatments
described above, the support can be pretreated by applying a
hydrophilic polymer thereto which acts as a hydrophilic
30 subbing layer and which improves adhesion of the disposed
composition described above. Subbing layer polymers and
methods for their preparation are well known in the art.
Generally, such polymers are composed of recurring units
having one or more pendant anionic or hydrophilic groups
35 such as carboxy, sulfonyl, phosphono, phosphinyl, carbonyl

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and hydroxy. Other general characteristics of such polymers include, but are not limited to, the presence of some halogen content from monomers such as vinyl chloride, vinylidene dichloride and others readily apparent to one skilled in the art.

Particularly useful subbing layer materials include poly(acrylonitrile-co-vinylidene chloride-co-acrylic acid), poly(methyl acrylate-co-vinylidene chloride-co-itaconic acid), poly(monomethyl itaconate-co-vinylidene chloride), poly(monoethyl itaconate-co-vinylidene chloride) and poly(monobutyl itaconate-co-vinylidene chloride).

The element of this invention can be prepared by disposing the nucleic acid reagent composition on a sealable support as defined herein, and heating the disposed composition at a temperature and for a time sufficient to dry the composition. While the time and temperature for suitable adhesion can be varied inversely, in general, a temperature in the range of from 10 to 80°C less than the glass transition temperature of the first polymer is used. The time for heating is generally from 10 to 40 seconds.

The element of this invention can be used in the analytical methods described in more detail below and in a number of formats. For example, the element can be merely a simple article which is used in a laboratory or doctor's office with standard pipettes, beakers and other equipment known for such assays, including a suitable container in which the assay is carried out. The most crude container would be a test tube, cuvette, flask or beaker, but more sophisticated containers have been fashioned in order to facilitate automated procedures for performing the method.

Alternatively, the element can be incorporated and used as part of a disposable test device

A preferred container for incorporation of the element is a self-contained test device like those described in EP-A-0 381 501. Such test devices are also known in the

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art as chemical test packs, pouches or cuvettes. They are constructed to provide certain temperature characteristics during the practice of the method as described in US-A-4,902,624. Such test devices have a multiplicity of
5 reaction chambers (or "blisters" as they are often called) having various reagents, buffers and other materials which are useful at various stages in the assay. The test devices can be appropriately and rapidly heated and cooled in cycles to promote the various steps of PCR amplification, a
10 preferred method of this invention. Other useful containers could be suitably fashioned for automated or single use of the method of this invention.

In such test devices, the element of this invention is disposed in a particular chamber (or "blister") through
15 which reagents and fluids are passed in order to facilitate a sequence of reactions in order to obtain detection of the target nucleic acid. In this instance, the reagents and fluids are considered to "flow by" the immobilized nucleic acid reagent on the element. The nucleic acid reagent on
20 the element is used to "capture" the target nucleic acid for detection. The element can be incorporated into the test device by affixing it with adhesives, fusing the element to a polymeric wall of the test device or using other
25 conventional means. In another embodiment, the element itself can form a wall or integral portion of the test device.

The test device can be self-contained, meaning that, while it has means for cooperating with outside sources of pressure for liquid transfer, it is constructed
30 to be completely closed after a sample has been introduced so that no leakage can occur.

In a preferred embodiment, the test device comprises two relatively thin sheets of flexible polymeric material [such as a heat- or pressure-sealable polyethylene-
35 poly(ethylene terephthalate) laminate, for example

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SCOTCHPAK™ heat-sealable film No. 229 or No. 241 both available from 3M Corporation] which are secured along their outer edges using heat or ultrasonic pressure-sealing. A heat-activatable adhesive, such as copolyesters of ethylene glycol, diethylene glycol and terephthalic acid as described in US-A-4,352,925, or a mixture of a polystyrene-polyisoprene-polystyrene block copolymer and a poly(ethylene-co-vinyl acetate) as described in US-A-4,126,464 can be used also.

10 Chambers or channels are formed between the sheets by sealing the sheets at certain points so that reagents and fluids can be kept within the test device and moved therethrough using pressure devices. Within one such chamber is located the element of this invention.

15 Preferably, the element is located in a chamber which is designed in a relationship with the other compartments and passageways so that the reagents and fluids used in the method, flow by the element and into a storage chamber designated to collect waste reagents and fluids.

20 The element of this invention can be used in a wide variety of methods for the detection of a target nucleic acid. It is particularly useful for the amplification and detection of one or more specific nucleic acid sequences present in one or more target nucleic acids in a test specimen. Such specimens can include cellular or viral material, hair, body fluids or other materials containing genetic DNA or RNA which can be detected.

The present invention is especially useful for producing, in exponential quantities relative to the number of reaction steps involved, at least one specific nucleic acid sequence of an infectious agent. The product will be a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed. Any source of nucleic acid, purified or not, can be utilized as the starting material. A mixture of nucleic acids can be

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employed if desired. The sequence to be duplicated can be a fragment of the entire nucleic acid. Moreover, a plurality of double stranded nucleic acids can be amplified and detected simultaneously by using a corresponding set of
5 primers and detection means (including capture reagents described above) for each specific nucleic acid. Multiple sequences in the same nucleic acid can also be amplified and detected.

Nucleic acids to be detected can be obtained from
10 various sources including plasmids, naturally occurring DNA or RNA from any source. It may be extracted from various tissues including blood, peripheral blood mononuclear cells (PBMC), tissue material or other sources known in the art using known procedures. The present invention is
15 particularly useful for the amplification and detection of nucleic acid sequences found in genomic DNA, bacterial DNA, fungal DNA, viral RNA, or DNA or RNA found in bacterial or viral infected cells.

The method described herein can be used to provide
20 the detection or characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancers. It may also be used in forensic investigations and DNA typing.

In one embodiment, the element is used to provide
25 a capture probe in what is known as a hybridization assay. Details of hybridization assays, procedures and reagents useful therein are described in such references as US-A-4,358,535, US-A-4,486,539 and US-A-4,925,785.

The elements of this invention are also useful for
30 "capturing" amplified targeted nucleic acids following polymerase chain reaction (or PCR). The details of PCR are also widely known. Detection probes useful in the practice of this invention can be hybridized directly or indirectly to the targeted nucleic acid. By "indirectly" is meant that
35 there may be intermediate oligonucleotides which are

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complementary to both the target nucleic acid and the detection probe. Procedures for attaching labels and preparing probes are well known in the art. Useful labels include radioisotopes, electron-dense reagents, chromogens, fluorogens, phosphorescent moieties, ferritin and other magnetic particles, biotin and other specific binding moieties, chemiluminescent moieties and enzymes. Useful enzymes include, but are not limited to, glucose oxidase, peroxidases, uricase, β -galactosidase, glycosylase and alkaline phosphatase, and can be attached to oligonucleotides using known procedures. Substrates and dye forming compositions for such enzymes are well known.

Where the label is a more preferred enzyme such as a peroxidase, at some time during the assay, hydrogen peroxide and suitable dye-forming compositions are added to provide a detectable dye. For example, useful dye-providing reagents include tetramethylbenzidine and derivatives thereof, and leuco dyes, such as triarylimidazole leuco dyes, or other compounds which react to provide a dye in the presence of peroxidase and hydrogen peroxide. Particularly useful dye-providing compositions are described in US-A-5,024,935.

In an alternative and preferred embodiment, a primer of the PCR process is biotinylated and the amplified nucleic acid is detected using detectably labeled avidin or a derivative thereof. For example, avidin can be conjugated with an enzyme, or have a radioactive moiety or another detection moiety like those described for the detection probes noted above. Biotin on the amplified product complexes with the avidin, and appropriate detection reagents and techniques are used. Biotinylation of primers can be carried out using known procedures.

The element of this invention can be included in a diagnostic kit which also contains one or more reagents,

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equipment or instruction sheets for using the element. Preferably, the kit includes a detection reagent for detecting the hybridization product of the nucleic acid reagent oligonucleotide and target nucleic acid in the element and the target nucleic acid (either with or without PCR). By "detection reagent" is meant one or more individual reactive materials which can singly or collectively provide a detectable signal. Most preferably, the detection reagent is a biotinylated primer used in PCR which is complementary to the target nucleic acid. Alternatively, the detection reagent is a water soluble, labeled oligonucleotide useful in a hybridization assay.

The kit can include other reagents and fluids such as, but not limited to, wash solutions, extraction compositions, diluents, buffers, primers, probes, DNA polymerases and PCR reagent compositions. The kit components are generally packaged individually in a suitable manner for transport and storage under the appropriate conditions.

The following examples are included to illustrate the practice of this invention, and are not meant to be limiting in any way. All percentages are by weight unless otherwise noted.

Materials and Methods for Examples:

A nucleic acid reagent used in Examples 1 and 2 was prepared as follows:

Polymeric particles (1.2 μ m average diameter) of poly[styrene-co-mono-2-(p-vinylbenzylthio)ethyl succinate] (95:5 weight ratio) were prepared using known emulsion polymerization techniques. To these particles were attached molecules of the oligonucleotide:

SEQ ID NO:1:

5'-X-GAGACCATCA ATGAGGAAGC TGCAGAAT-3'.

wherein X is an aminediol linking group with two tetraethylene glycol spacer groups prepared and attached to

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the oligonucleotide according to the teaching of US-A-4,962,029.

The oligonucleotide molecules were attached to the particles to form a nucleic acid reagent as follows:

5 The particles were washed twice with 2-(N-morpholino)ethanesulfonic acid buffer (0.1 molar, pH 6).
- Samples (30 µg) of the particles suspended in the buffer (1 ml) were mixed with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.15 ml of 100 mg/ml of
10 buffer) and the oligonucleotide (0.0288 ml of 57.3 OD/ml of purified water, 1.65 OD units or 55 µg). The mixture was rotated end over end at room temperature (20-25°C) for 15 hours and centrifuged. The particles were washed three times with water and resuspended in purified water at a
15 solids content of 0.9%.

 The nucleic acid reagent was mixed with an adhesive of poly(methyl acrylate-co-2-acrylamido-2-methylpropanesulfonic acid, sodium salt-co-2-acetoacetoxyethyl methacrylate) (90:4:6 weight ratio). The
20 resulting composition comprised about 18% (dry weight) of adhesive. This mixture, in aqueous suspension, was applied to a heat-sealable support to form an element as described below in Example 1.

 Primers used in the PCR process of Example 2 were
25 as follows:

SEQ ID NO:2:

5'-X-AGTGGGGGGA CATCAAGCAG CCATGCAAA-3'

SEQ ID NO:3:

5'-TTCCTGCTAT GTCACCTCCC CTTGGTTC-3'

30 wherein X represents a biotintetraethylene spacer group prepared and attached to the oligonucleotide according to the teaching of US-A-4,914,210.

 For Example 3, the nucleic acid reagent was similarly prepared using particles of poly[styrene-co-3-(p-vinylbenzylthio)propionic acid] (97.6:2.4 molar ratio) of
35

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different sizes, and the following oligonucleotide was similarly attached to the particles:

SEQ ID NO:4:

5'-ATTATCTTTT ATATTCTAAC CAGGATT-X-3'

5 wherein X comprises two tetraethylene glycol spacers and a 3-amino-1,2-propanediol linker at the 3' end.

Particles of poly[m&p-vinyltoluene (64:36)-co-methacrylic acid] (98:2 weight ratio) were also used in Example 3. These particles were prepared either using
10 conventional suspension polymerization techniques or the limited coalescence procedures described in US-A-3,615,972.

The target nucleic acid for Example 2 was a 140-nucleotide segment of the gag region of HIV-I.

A wash solution (250 μ l) comprised a buffered
15 solution of sodium phosphate (10 mmolar, pH 7.4), sodium chloride (150 mmolar), ethylenediaminetetra-acetic acid (1 mmolar) and decyl sulfate (1%). The wash solution was preheated to 55°C for use.

The DNA polymerase used in the PCR was isolated
20 from Thermus aquaticus.

A conjugate of streptavidin and horseradish peroxidase was obtained from Zymed Labs (San Francisco) and was diluted 1:8000 with casein (0.5%) in a phosphate buffer solution (pH 7.3) containing thimerosal preservative
25 (0.01%).

A leuco dye composition was prepared as follows:
2-(4-hydroxy-3,5-dimethoxyphenyl)-4,5-bis(4-methoxyphenyl)imidazole (to make a 0.1% solution) was dissolved in a solution of poly(vinyl pyrrolidone) (20%) in
30 sodium phosphate buffer (5 mmolar). This solution was then added to a solution containing hydrogen peroxide (10 mmolar), 4'-hydroxyacetanilide electron transfer agent (5 mmolar) and diethylenetriamine-pentaacetic acid chelating agent (10 μ molar) in sodium phosphate buffer to produce a

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final concentration of 1% poly(vinylpyrrolidone) and 0.005% leuco dye.

The PCR reaction solution for Example 2 comprised a solution of tris(hydroxymethyl)amino-methane buffer (10 mmolar, pH 8), potassium chloride (50 mmolar), magnesium chloride (10 mmolar) and gelatin (10 µg). To this solution were added the primers (100 pmoles of each), dNTP's (1.5 mmolar of each) and the DNA polymerase (7.5 units).

For Example 3, the following solutions were prepared to provide the PCR reagents:

A storage buffer for the DNA polymerase was prepared from tris(hydroxymethyl)aminomethane buffer (8 ml, 1 molar, pH 8), potassium chloride (13.32 ml, 3 molar), diethiothreitol (0.4 ml, 1 molar), ethylenediaminetetraacetic acid (0.16 ml, 0.25 molar), glycerol (250 ml, 80%), a surfactant solution (6.67 ml) containing 30% NONIDET™ P-40 nonionic surfactant (Sigma Chemical) and 30% TWEEN™ 20 nonionic surfactant (ICI Americas, Inc.) and a solution of gelatin (4 ml, 2%) so that the final concentrations were buffer (20 mmolar), potassium chloride (100 mmolar), dithiothreitol (1 mmolar), ethylenediaminetetraacetic acid (0.1 mmolar), glycerol (50%), surfactants (0.5% each), gelatin (200 µg/ml) and water to make 400 ml of solution.

A second buffer solution was also prepared to contain tris(hydroxymethyl)aminomethane (10 mmolar, pH 8.3), potassium chloride (50 mmolar), magnesium chloride (10 mmolar), gelatin (10 µg) and glycerol (7.5%).

A third buffer solution was prepared containing tris(hydroxymethyl)aminomethane (10 mmolar) and TWEEN™ 20 nonionic surfactant (0.5%).

The PCR reagent solution was then prepared from the above three buffer solutions by adding a mixture of 12 µl of the storage buffer to 288 µl of the second buffer solution, then to 220 µl of this mixture, adding 80 µl of

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the third buffer solution, and finally adding the biotinylated target nucleic acid thereto to a concentration of 40 pmolar.

Example 1 Preparation of Element

5 This example demonstrates the preparation of an element of this invention and compares it to an element outside the scope of this invention, that is, an element wherein the nucleic acid reagent is adhered to a support in another fashion.

10 An element of this invention was prepared using as a solid support a sample of Type 241 SCOTCH PAK™ laminate (3M Company) which is a laminate of polyethylene and polyethylene terephthalate. The polyethylene side of the laminate was corona treated to a level of 56-59 dynes/cm²
15 using conventional treatment equipment obtained either from Pillar Technologies (Hartland, Wisconsin) or Enercon Industries (Menomonee, Wisconsin).

 The polyethylene side of a sheet of polyethylene-poly(ethylene terephthalate) laminate was heat sealed over
20 the element support to form an enclosed detection channel. This channel contained an inlet tip for injection of fluids into the channel and an outlet means to allow fluids to exit the channel.

 The composition of adhesive and nucleic acid
25 reagent described above was applied to the corona treated support in the form of an aqueous suspension (2 µl, 0.9% solids of reagent, 0.2% adhesive) in water. The suspension was applied in a defined region of the support in the detection channel and dried using a hot iron (95°C for 30
30 seconds) in contact with the bottom of the support. The sheet of laminate was then heat sealed over the element to form an enclosed detection channel.

 Before heat sealing the sheet of laminate over the element, a physical test was performed to determine how well
35 the nucleic acid reagent composition was immobilized onto

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the support. This test comprised rubbing the dried deposit of composition, and folding the support 180 degrees. In the element of this invention, the reagent did not detach from the support but was firmly immobilized thereon. -Very little
5 composition came off the support during these physical tests.

A Control element was similarly prepared using the same support and nucleic acid reagent except that the adhesive was omitted. The nucleic acid reagent (0.9% solids
10 suspension) in 1 μ l glycine buffer (0.1 molar, pH 8.5) was applied to a defined region of the corona treated support, dried and a sheet of laminate was heat sealed over the deposit as described above. Before heat sealing, the dried
15 reagent was physically rubbed and the support was bent 180 degrees to see how well the reagent adhered to the support. Much of the reagent detached from the support during these physical tests.

Example 2 Use of Elements in PCR Method

This example demonstrates and compares the use of
20 the elements described in Example 1 in the amplification and detection of a sequence of HIV-I DNA.

To the PCR reagent solution described above were added different amounts of the target HIV-I DNA fragment noted above (0, 100, 10^3 and 10^4 copies or molecules). The
25 total volume of each of the resulting solutions was 100 μ l.

Each solution was placed in 250 μ l microfuge tubes and amplified using PCR through 40 cycles of the following protocol:

30 Denaturation: heat to 96°C and hold for 30 seconds, and

 Priming/extension: cool to 68°C and hold for 1 minute.

 The apparatus used for the PCR was a Perkin-Elmer Thermocycler commercially available from Cetus/Perkin Elmer.

 After the last cycle, the solutions were heated to
35 95°C and held for 5 minutes to denature the resulting

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double-stranded products. The heated solutions were each injected into the detection channel of the elements (Example 1 and Control) in a manner to insure uniform contact with the nucleic acid reagent immobilized therein. Following
5 solution injection, each element was incubated at 42°C for 5 minutes to allow hybridization of the complementary target nucleic acid to the oligonucleotide of the reagent in the element. Remaining fluid was removed either by forcing it out with compressed air, or by drawing it out using a
10 syringe.

The wash solution described above was injected into each element twice to remove unhybridized materials. Once the wash fluid had been removed the last time, the solution containing the conjugate of streptavidin and
15 peroxidase (200 µl) was injected into each element and incubated at 42°C for two minutes. Once that solution was removed, the leuco dye solution (200 µl) was similarly injected followed by another incubation for five minutes.

The remaining fluid was removed, and the dye
20 signal apparent in the element was visually graded on a scale of 0 to 10 (highest density). The results are shown in the FIGURE. The background (0 copy number) for both elements was quite low. However, for the assays of amplified target nucleic acid, the element of this invention
25 provided higher signal at each concentration of target nucleic acid, indicating that the nucleic acid reagent was better retained in the element through the detection process with its repeated washes and contact with several different fluids. As noted above in Example 1, much of the reagent in
30 a sample of the Control element was easily rubbed off the support, so it is believed that the fluids used in the assay removed it during the assay. It is quite important that the element of this invention demonstrated high sensitivity to the presence of a low concentration (100 copy number) of
35 target nucleic acid.

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Example 3 Comparative Example Using Mixture of
Different Sized Particles in Reagent Using
Treated and Untreated Supports

5 This example demonstrates the practice of this invention in preparing an element containing a nucleic acid reagent composition, and compares its use to the use of an element outside the scope of this invention. It also compares the use of treated supports to the use of untreated
10 supports.

 Poly[styrene-co-3-(p-vinylbenzylthio)-propionic acid] (mole ratio 97.6:2.4, weight ratio 95:5) aqueous polymer bead dispersions were prepared with different particle sizes, and an oligonucleotide identified below was
15 covalently bound thereto. The oligonucleotide had the sequence SEQ ID NO:4 shown above linked to the particle through two tetraethylene glycol spacers, a 3-amino-1,2-propanediol moiety, and a thymine base. The oligonucleotide was appended to the polymer particle through the amino group
20 of the 3-amino-1,2-propanediol moiety to form reagents.

 Reagents were prepared having different average particle sizes:

 Reagents useful in the practice of this invention comprised particles having an average size of about 1.3 μ m
25 and were formulated at 2.0% solids with the polymeric adhesive described above (0.1% solids) in 2-amino-2-hydroxymethyl-1,3-propanediol/ethylenediamine tetraacetic acid buffer.

 Nucleic acid reagents outside the scope of this
30 invention (Control A) comprised particles having an average size of about 8 μ m and were formulated at 4.0% solids with the polymeric adhesive (0.1% solids) in 2-amino-2-hydroxymethyl-1,3-propanediol/ethylene-diamine tetraacetic acid buffer. Controls B and C contained mixtures of
35 particles with different sizes: Control B was a mixture of 1.3 μ m particles (20%) and 8.0 μ m particles (80%), and

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Control C was a 50:50 mixture (weight) of particles of the two sizes.

The reagent formulations were used to prepare the following elements having the reagents as capture probes for an assay:

| Variation | 1 | 2 | 3 | 4 | Corona Treatment? |
|-----------|-----------|-----------|-----------|-----------|-------------------|
| 1 | Invention | Control A | Control B | Control C | No |
| 2 | Invention | Control A | Control B | Control C | Yes - 8 seconds |

The elements were prepared by heating a sheet of poly(ethylene terephthalate)/polyethylene laminate (SCOTCHPAK™ 241, 3M Co.) at a forming station (or mold) to form an array of depressed areas toward one side of the sheet, so that upon lamination to a cover sheet at a later time, the resulting pouch had narrow channels. Each depressed area was later filled with an appropriate reagent composition. A sheet was laminated to form a cover over the depressed and channel areas, and sealed to create a burst seal between each depressed area and the channel leading from it to the main channel. First, however, one section near the end of the main channel was treated with corona discharge from an Enercon DYNE-A-MITE™ corona discharge unit at 0.95 cm clearance to a TEFLON™ dielectric plate for 8 seconds. The reagent formulations described above (Invention & Controls A-C) were then immediately deposited in four spots on the treated surface to form an element, each spot having 0.9 to 1.1 μ l of formulations noted above, in a row. The disposed formulations were dried for about 20 seconds in a stream of air at room temperature while heating the opposite side of the support with an iron at about 95°C. Another set of elements were similarly prepared but without the corona discharge treatment.

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The blisters (or chambers) formed in the device were filled with the desired reagents. A first blister of each element was adapted to receive a specimen to be tested and to hold it during heating. A second blister was filled with a streptavidin-horseradish peroxidase conjugate composition as described above. A third blister contained a 1% sodium decylsulfate wash solution (in phosphate buffered saline solution containing 10 mmolar sodium phosphate, 150 mmolar sodium chloride and 1 mmolar ethylenediaminetetraacetic acid, pH 7.4), and a fourth blister contained the leuco dye composition described above. A last blister located at the end of the main channel was larger than the others and fitted with an absorbent to be a reservoir for waste fluids.

The cover sheet was then laminated and sealed in three steps. First the sandwich was pressed and sealed by heating at about 149°C only around the blisters containing the 3 reagent solutions and around the waste blister. The formation of the sample-receiving PCR blister, including burst seals, and the channels was completed by heating the test pack between appropriately shaped heating jaws at about 163°C. The third step was the formation of perimeter seals around the test pack, and resealing all blister perimeter seals using a top plate temperature of 199°C while the bottom plate remained at ambient temperature. The channels formed in the completed test pack (or element) were located so that passage of a roller across the portion of the element containing the blisters would sequentially burst the seals of the blisters and force the reagent from each blister into and along an exit channel to the main channel leading to the area containing the capture probes.

The completed integral test elements were used to evaluate the reagent formulations as follows:

A blister in the test device was filled with about 205-210 µl of a solution of a biotinylated oligonucleotide

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reagent used as a target nucleic acid which had a nucleotide sequence complementary to that of the oligonucleotide covalently bound to the polymer particles (that is, the nucleic acid reagent), and sealed. The sequence of that
5 biotinylated target nucleic acid was as follows:
- SEQ ID NO:5:

5'-X-AATCCTGGTT AGAATATAAA AGATAAT-3'

wherein X is a single tetraethylene glycol linking group for attachment to biotin. The concentration of the target
10 nucleic acid in the PCR reaction solution was believed to be 40 pmolar.

The blister containing this reagent was preheated to 95-98°C for 120 seconds and then rolled to break the seal and advance the solution to the area containing the nucleic
15 acid reagent. The oligonucleotide reagent and capture reagent were hybridized in the detection blister during incubation at 42°C for 5 minutes while the blister containing the conjugate of avidin-horseradish peroxidase was heated to 35°C. The seal of the conjugate blister was
20 broken, and the solution directed to the detection area, where it immunologically reacted with the available biotinylated reagent. The seal of the wash solution blister (preheated to 55°C for 300 seconds) was broken and the wash solution directed to the detection area to clean out the
25 main channel and to remove unbound conjugate from the detection area. The seal of the blister containing the dye-forming composition was then broken and the composition directed to the detection area. Incubation for 5 minutes proceeded to allow for dye formation before reading the
30 color density scores.

The color density scores, provided in Table I below, were evaluated for two features: "quality of dye spot" and "color of dye spot".

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The "quality of dye spot" was judged on a 1 to 5 score basis with each score having the following evaluation (with the lower scores being desired):

- 5 1 - Good spot, no flaking or defects visually detected,
- 2 - Pitting or very slight flaking (less than 25% of spot),
- 3 - Slight flaking (about 25% of spot),
- 4 - Significant flaking (25-75% of spot),
- 10 5 - Spot completely flaked off.

 The "color of dye spot" was evaluated by visual comparison of the wet dye density to a color chart, where 0 is no density and 10 is highest density. A high score is desirable here. The aggregate of the "quality" and "color" 15 scores indicates whether the reagent formulation improved adhesion ("quality"), detracted from signal ("color") generated, or both.

Table I
"Quality of Dye Spot"

20

| Variation | 1 (Invention) | 2 (Control A) | 3 (Control B) | 4 (Control C) |
|-----------|------------------|------------------|------------------|------------------|
| 1 (dry) | 1.17 | 2.08 | 1.25 | 1.83 |
| 1 (wet) | 4.08 | 4.75 | 4.66 | 4.25 |
| 2 (dry) | 1.00 | 1.00 | 1.00 | 1.08 |
| 2 (wet) | 1.08 | 1.83 | 1.58 | 1.83 |

"Color of Dye Spot"

| Variation | 1 (Invention) | 2 (Control A) | 3 (Control B) | 4 (Control C) |
|-----------|--|---|---|---|
| 1 | 6.0 (9 of 12 spots washed away) | 5.33 (9 of 12 spots washed away) | 6.0 (10 of 12 spots washed away) | 6.80 (7 of 12 spots washed away) |
| 2 | 7.42 | 6.67 | 6.92 | 7.75 |

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The conclusions reached from the data presented in Table I include:

- 1) The elements having non-corona treated supports exhibited acceptable dye density (that is, sensitivity), but many reagents were washed away (that is, insufficient adhesion).
- 2) The reagent formulations containing larger particles generally were more opaque and provided lower spot quality and color.
- 3) The best "quality of dye spot" score was achieved with the small particle reagent formulation (both wet and dry).
- 4) The mixture of beads (two sizes) generally failed to provide the desired combination reagent adhesion and dye density.

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SEQUENCE LISTING

(1) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 28 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Probe oligonucleotide

10 (iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same

(x) PUBLICATION INFORMATION: None

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAGACCATCA ATGACGAAGC TGCAGAAT

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 29 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA Primer

25 (iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(vi) ORIGINAL SOURCE: Synthetically prepared

(vii) IMMEDIATE SOURCE: Same prepared

(x) PUBLICATION INFORMATION: None

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGTGGGGGGA CATCAAGCAG CCATGCAAA

(3) INFORMATION FOR SEQ ID NO:3:

- 35 (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 28 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
- 5 (ii) MOLECULE TYPE: DNA Primer
- (iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
(vii) IMMEDIATE SOURCE: Same prepared
10 (x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
TTCCTGCTAT GTCACCTCCC CTTGGTTC
- 15 (4) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
20 (D) TOPOLOGY: Linear
(ii) MOLECULE TYPE: Probe oligonucleotide
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No
(vi) ORIGINAL SOURCE: Synthetically prepared
25 (vii) IMMEDIATE SOURCE: Same prepared
(x) PUBLICATION INFORMATION: None
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
ATTATCTTTT ATATTCTAAC CAGGATT
- 30 (5) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 nucleotides
(B) TYPE: Nucleic acid
(C) STRANDEDNESS: Single
35 (D) TOPOLOGY: Linear

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- (ii) MOLECULE TYPE: Oligonucleotide
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: No
- (vi) ORIGINAL SOURCE: Synthetically prepared
- 5 (vii) IMMEDIATE SOURCE: Same prepared
- (x) PUBLICATION INFORMATION: None
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AATCCTGGTT AGAATATAAA AGATAAT

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Claims:

1. An element comprising a sealable support having disposed thereon a nucleic acid reagent composition comprising a mixture of:
 - 5 a. a nucleic acid reagent comprising:
particles composed of a first polymer having a glass transition temperature of at least 70°C, said particles having an average diameter of from 0.1 to 3 micrometers, and
10 an oligonucleotide covalently attached to said particles, and
 - b. a water insoluble adhesive comprising a second polymer which has a glass transition temperature which is at least 30°C less than the glass transition temperature of
15 said polymeric adhesive being present in said composition at from 1 to 20 weight percent.
2. The element of claim 1 wherein said support has hydrophilic surface groups provided by corona discharge treatment, chromic acid treatment or treatment with a radio
20 frequency electromagnetic field in the presence of a reactive gas or by having thereon a hydrophilic subbing layer on which said composition is disposed.
3. The element of claim 1 wherein the average diameter of said polymeric particles is from 0.5 to 2
25 micrometers.
4. The element of claim 1 wherein said first polymer glass transition temperature is from 70 to 175°C, and said second polymer glass transition temperature is from
30 30 to 120°C less than said first polymer glass transition temperature.
5. The element of claim 1 wherein said adhesive is present in said composition at from 2 to 10 weight percent.
6. The element of claim 1 wherein said first
35 polymer comprises:

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- a. from 0.1 to 60 weight percent of recurring units derived from one or more ethylenically unsaturated polymerizable monomers having a reactive group selected from the group consisting of an active halogen, carboxy, amidine, 5 activated 2-substituted ethylsulfonyl, activated 2-substituted ethylcarbonyl, an active ester, vinylsulfonyl, vinylcarbonyl, aldehyde, hydrazine, epoxy, amino and sulphydryl,
- b. from 60 to 99.9 weight percent of recurring units 10 derived from one or more ethylenically unsaturated polymerizable monomers which provide hydrophobicity, and
- c. from 0 to 15 weight percent of recurring units derived from one or more ethylenically unsaturated polymerizable monomers other than those defined in (a) and 15 (b).
7. * The element of claim 1 wherein said second polymer comprises:
- d. from 55 to 100 weight percent of recurring units derived from one or more ethylenically unsaturated 20 polymerizable monomers which provide hydrophobicity,
- e. from 0 to 45 weight percent of recurring units derived from one or more ethylenically unsaturated polymerizable monomers which form a water soluble homopolymer or which provide hydrophilicity, and
- 25 f. from 0 to 15 weight percent of recurring units derived from one or more ethylenically unsaturated polymerizable monomers which provide crosslinking in said second polymer.
8. The element of claim 1 wherein said 30 composition is disposed in a defined region of said support.
9. A self-contained test device comprising an element comprising a sealable support having disposed thereon a composition comprising a mixture of:
- a. a nucleic acid reagent comprising:

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particles composed of a first polymer having a glass transition temperature of at least 70°C, said particles having an average diameter of from 0.1 to 3 micrometers, and an oligonucleotide covalently attached to said particles,

5 and

b. a water insoluble adhesive comprising a second polymer which has a glass transition temperature which is at least 30°C less than the glass transition temperature of said first polymer,

10 said polymeric adhesive being present in said composition at from 1 to 20 weight percent.

10. A method for preparing a nucleic acid test element comprising:

15 A. depositing a nucleic acid reagent composition on a sealable support, said composition comprising a mixture of:

a. a nucleic acid reagent comprising:

20 particles composed of a first polymer having a glass transition temperature of at least 70°C, said particles having an average diameter of from 0.1 to 3 micrometers, and

an oligonucleotide covalently attached to said particles, and

25 b. a water insoluble polymeric adhesive comprising a second polymer which has a glass transition temperature which is at least 30°C less than the glass transition temperature of said first polymer,

said polymeric adhesive being present in said composition at from 1 to 20 weight percent, and

30 B. heating said deposited nucleic acid reagent composition at a temperature and for a time sufficient to adhere said composition to said support.

11. A diagnostic test kit comprising:

(1) an element comprising a sealable support having disposed thereon a composition comprising a mixture of:

35 a. a nucleic acid reagent comprising:

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particles composed of a first polymer having a glass transition temperature of at least 70°C, said particles having an average diameter of from 0.1 to 3 micrometers, and an oligonucleotide covalently attached to said particles, which oligonucleotide is complementary to a targeted nucleic acid,

b. a water insoluble adhesive comprising a second polymer which has a glass transition temperature which is at least 30°C less than the glass transition temperature of said first polymer,

said polymeric adhesive being present in said composition at from 1 to 20 weight percent, and

(2) a detection reagent for detecting the hybridization product of said nucleic acid reagent oligonucleotide in said element and said target nucleic acid.

12. The kit of claim 11 wherein said detection reagent is a biotinylated primer which is complementary to said target nucleic acid.

13. A method for the detection of a target nucleic acid comprises:

A. contacting a specimen suspected of containing a target nucleic acid with an element comprising a sealable support having disposed thereon a nucleic acid reagent composition comprising a mixture of:

a. a nucleic acid reagent comprising: particles composed of a first polymer having a glass transition temperature of at least 70°C, said particles having an average diameter of from 0.1 to 3 micrometers, and an oligonucleotide covalently attached to said particles, said oligonucleotide being complementary to said target nucleic acid, and

b. a water insoluble adhesive comprising a second polymer which has a glass transition temperature

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which is at least 30°C less than the glass transition temperature of said first polymer,

said polymeric adhesive being present in said composition at from 1 to 20 weight percent,

5 to form a hybridized product of said target nucleic acid and said nucleic acid reagent on the element, and

- B. detecting the presence of said hybridized product as an indication of the presence of said target nucleic acid in said specimen.

10 14. A method for the amplification and detection of a target nucleic acid comprises:

 A. amplifying a target nucleic acid in a test specimen,

 B. contacting said amplified target nucleic acid with
15 the element comprising a sealable support having disposed thereon a nucleic acid reagent composition comprising a mixture of:

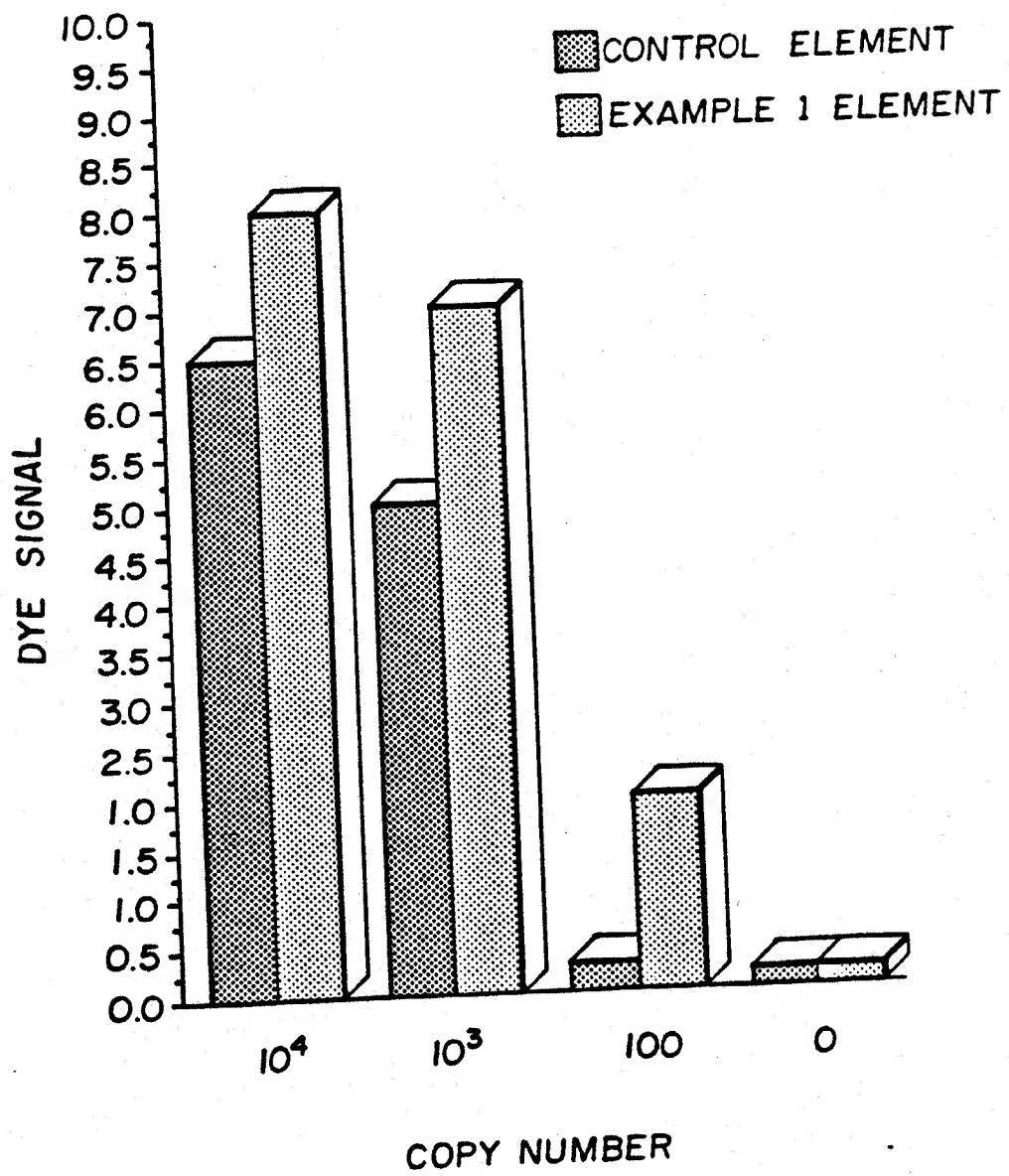
 a. a nucleic acid reagent comprising:
particles composed of a first polymer having a glass
20 transition temperature of at least 70°C, said particles having an average diameter of from 0.1 to 3 micrometers, and
an oligonucleotide covalently attached to said particles, said oligonucleotide being complementary to said target nucleic acid, and

25 b. a water insoluble adhesive comprising a second polymer which has a glass transition temperature which is at least 30°C less than the glass transition temperature of said first polymer,

 said polymeric adhesive being present in said
30 composition at from 1 to 20 weight percent,

 to form a hybridized product of said amplified target nucleic acid and said nucleic acid reagent on the element,
and

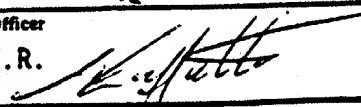
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INTERNATIONAL SEARCH REPORT

PCT/US 92/02200

International Application No

| | | |
|---|---|-------------------------------------|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)* | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC | | |
| Int.Cl. 5 C12Q1/68; //G01N33/543 | | |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁷ | | |
| Classification System | Classification Symbols | |
| Int.Cl. 5 | C12Q ; B01L ; G01N | |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ | | |
| Category ⁹ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| A | US,A,4 790 640 (NASON F.L.) 13 December 1988 see column 4, line 24 - line 41 see column 9, line 21 - line 49 --- | 1 |
| A | EP,A,0 267 724 (UNILEVER PLC) 18 May 1988 see column 2, line 34 - line 54; example 1 see column 4, line 33 - line 58; claims --- | 1 |
| T | EP,A,0 478 047 (EASTMAN KODAK COMPANY) 1 April 1992 --- | |
| <p>* Special categories of cited documents : ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report | |
| 28 JULY 1992 | 10.08.92 | |
| International Searching Authority | Signature of Authorized Officer | |
| EUROPEAN PATENT OFFICE | LUZZATTO E.R.  | |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9202200
SA 58729**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 28/07/92

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| US-A-4790640 | 13-12-88 | None | |
| EP-A-0267724 | 18-05-88 | AU-B- 613325 | 01-08-91 |
| | | AU-A- 8153787 | 25-05-88 |
| | | DE-A- 3777085 | 09-04-92 |
| | | WO-A- 8803270 | 05-05-88 |
| | | JP-T- 1501097 | 13-04-89 |
| EP-A-0478047 | 01-04-92 | None | |